

Professor A. P. Hill,
with the kind regards
of John J. Abel

ON THE UNITARY VERSUS THE MULTIPLE HORMONE
THEORY OF POSTERIOR PITUITARY PRINCIPLES

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ON THE UNITARY VERSUS THE MULTIPLE HORMONE THEORY OF POSTERIOR PITUITARY PRINCIPLES¹

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In 1923 with my associates, Rouiller and Geiling, I (1) published our last paper on the active principles of the infundibular portion of the pituitary gland and gave it as our opinion that "all the evidence at hand is greatly in favor of the belief that the oxytocic, pressor, diuretic and respiratory activities of posterior pituitary extracts are properties of one and the same substance." This is known as the unitary theory as distinguished from the belief that three or more separate principles exist as such in the posterior pituitary and are carried as separate entities in the blood stream. It will be admitted that it is a matter of importance to establish the validity of one or the other of these opposed theories. Does the posterior lobe, leaving out of consideration here its relation to the pars intermedia, send out into the blood stream a single, large, more or less unstable, molecule with multiple physiological activities, or does it yield three or more separate chemical compounds? This, it may be repeated, is the question at issue.

Since Oliver and Schafer (2) in 1895 first demonstrated the action of posterior pituitary extracts upon the vascular system physiologists and chemists have in general adopted the theory of a multiplicity of principles as most naturally accounting for the manifold activities of such extracts. The multiple theory has recently received great support in the work of Kamm, Aldrich, Grote, Rowe and Bugbee (3) who have improved on the methods employed by their predecessors and have succeeded, they believe, in obtaining "the substantially pure pressor principle in the form of a white, stable, water-soluble powder 80 times as potent as the

¹ An investigation carried out under a grant from the Carnegie Corporation of New York.

International Standard Powdered Pituitary" (and which also "has been shown to be responsible for the diuretic-antidiuretic action of pituitary extracts"); and "the separated oxytocic principle in the form of a white, stable, water-soluble powder which is more than 150 times as potent as the International Standard Powdered Pituitary."

In the past two years I have again occupied myself with these questions. In case we are dealing with a unitary substance, chemically unstable though it may be, methods may possibly be devised that will enable us to separate such a substance in a state of high purity from gland extracts. In case this cannot be achieved, and only a more or less complete differentiation or separation of three or more active principles is all that we can hope to offer, there would be little justification for maintaining the unitary hypothesis.

The following preliminary account of my work shows, however, that all of the known physiological activities of posterior lobe extracts, as far as assays have been made, are retained by my final "unitary" product.² Very naturally it will not be possible to give here more than the briefest outline of the methods employed.

METHODS

First step

Ammonium sulphate method. The fresh posterior lobes are ground up finely at the slaughter house with an excess of solid ammonium sulphate, after which a certain amount of saturated solution of the same salt is added, and the grinding is continued until a homogeneous semi-liquid paste is obtained. This paste is collected on a Büchner funnel and the residue is pressed as dry as possible and is then stirred up three times with appropriate quantities of a saturated solution of ammonium sulphate, filtered after each stirring and again pressed as dry as possible. The residue is now extracted three times with 60 per cent alcohol. The combined alcoholic extracts are filtered and to the clear filtrate abso-

² No assay of the antidiuretic property has yet been made, but it may be assumed to be a function of the unitary product, in view of the excellent therapeutic results that were obtained in 1923 by administration of one of my preparations of that time in several cases of diabetes insipidus. (See Abel and Geiling: Jour. Pharmacol. and Exper. Therap., 1923, xxii, 317.)

lute alcohol is added in small quantities at a time, to the extent of three volumes of absolute alcohol to one volume of the 60 per cent alcohol extract. The resulting voluminous precipitate is collected on Büchner funnels with gentle suction and finally kneaded and pressed on the funnel as dry as possible and set aside to dry in a current of air or in a desiccator.

When dry the precipitate is ground up with an appropriate volume of 90 per cent phenol and the resulting thick syrup or jelly is stirred with cold water until no further precipitate falls out. The precipitate adheres to the bottom and sides of the beaker as a gummy mass. After standing for some days the supernatant liquid, which is devoid of physiological activity, can be poured off and the precipitate dries to a brittle resinous mass which can be pried off from the bottom of the beaker with a sharp spatula and ground up to a fine powder. From 1500 grams of fresh posterior lobes one obtains in this way 50 grams of a dry powder which assays at about five times the standard pituitary powder. Naturally, a very much more active product, equal to about $10 \times$ the standard powder, is obtained when only the first extract with 60 per cent alcohol is utilized. The glandular residue left after the three extractions with 60 per cent alcohol is entirely devoid of physiological activity.

Alternative first step

After having employed the foregoing method for more than a year as the first step of my procedures I have now discarded it in favor of the following process, which involves less labor as it can be applied to the finely ground and dried posterior lobe preparation of commerce. A dry preparation of this kind, when of good quality, will assay 85 for pressor and oxytocic values as compared with 100 for the international standard powder; less carefully prepared powders may assay at not more than 50 per cent in terms of pressor and oxytocic action.

Twenty-five grams of the dried and finely powdered posterior lobes are ground up to a fine paste in a mortar, imbedded in ice, with a saturated solution of copper acetate in 4 per cent acetic acid (80 grams $\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$ in 800 cc. of 4 per cent acetic

acid). The cooled copper solution is added in small quantities at a time during the grinding process until 165 to 170 cc. have been added. The process is facilitated by the removal, from time to time, of the finely ground material from the upper part of the mortar. An equal volume of cold water is added to the ground-up mass and the mixture is then coagulated by the addition of 165 cc. of a saturated solution of sodium chloride. After allowing the mass to stand on ice for half an hour or more until flocculation is well advanced the heavy precipitate is collected on a suitable Büchner funnel at the suction pump and pressed as dry as possible. The compact cake is then ground up in a cooled mortar with 165 cc. of cold water, and the mixture, which is kept cold by means of ice, is treated with H_2S until it is permanently saturated with the gas. Before the H_2S is introduced, a few small droplets of caprylic alcohol are added in order to prevent foaming. Excess of H_2S is then removed by means of an air current. Cold absolute alcohol is added to the cold degassed mixture in small quantities at a time until the alcoholic content of the mixture amounts to 65 to 70 per cent. After allowing the flocculating CuS to subside, the 65 to 70 per cent alcoholic suspension of sulphide and insoluble proteins is filtered cold and washed with 65 to 70 per cent alcohol, and $2\frac{1}{2}$ volumes of absolute alcohol are added to the filtrate plus washings, in small portions at a time, followed by the addition of 1 to $1\frac{1}{2}$ volumes of ether. The resulting white, physiologically active precipitate is collected, washed thoroughly with acetone, forthwith ground up and dried in an air current. Assayed against a standard powder, this precipitate will be found to have a pressor and oxytocic value equal to from 6 to 9 times that of the standard powder, the variations in titre depending upon the relative excellence of the desiccated gland powder used in the process and the relative completeness of extraction. The operation described above is easily carried out and this first alcohol ether precipitate, as a rule, contains 70 per cent or more of the active constituents present in the starting material. The dry precipitate obtained in this way from 25 grams of dried posterior glands varies from 1.95 to 2.05 grams.

The considerable mass of tissue residues plus the CuS that has been extracted once with 65 to 70 per cent alcohol, as described above, retains the missing 30 per cent of activity. If it is thought desirable, this precipitate can be ground up with 160 cc. of 65 per cent alcohol, filtered under pressure, the insoluble residue washed with alcohol of the same strength, and pressed as dry as possible. The filtrate is precipitated as before with absolute alcohol and ether, washed with acetone and ground up in an air current until dry. This second extraction of the CuS precipitate yields an additional 16 to 20 per cent of active material. A third extraction yields only a relatively small amount of physiologically active material. The three extractions as described give a total yield of active material assaying at 95 to 97 per cent of that present in the desiccated product employed. For the time being, my associate, Dr. Joseph B. Koepfli, and I are limiting ourselves to the first extraction.

The above copper method has been described in sufficient detail to enable others to carry it out. By means of a single extraction, properly carried out, one is enabled to reduce 25 grams of a desiccated posterior lobe powder to 2 grams or thereabouts of a product whose assay value is 13 to 15 times greater than that of an equal weight of the original material if this be of good quality.

Further Procedure

Newer methods which have been elaborated for the further purification of the active unitary precipitate, as obtained by the use of the copper acetate method, are now being tested in my laboratory and will be described elsewhere in due season. I should like, however, to describe in the barest outline some results that were obtained a year or more ago by methods that were then applied to the further purification of an active powder, with an oxytocic and pressor value equal to 5 to 6 times that of the standard powder, which had been obtained from the fresh *pars posterior* by the now discarded ammonium sulphate method.

The reagents employed to carry this unitary substance with a value of, say, $5 \times$ the standard powder for both oxytocic and pressor

activity to the high level of $50 \times$ the standard powder for oxytocic, pressor and melanophore activities were: 90 per cent phenol (i.e., 90 parts of crystalline carbolic acid melted by gentle heat and then mixed with 10 parts of water), syrupy lactic acid (85 per cent lactic acid), acetic acid, alcohol, ether, acetone, phenyl acetate, petroleum ether, saturated aqueous solution of NaH_2PO_4 , NaCl . Some of these reagents were employed either as solvents or precipitants in accordance with the requirements of the situation, others, as NaH_2PO_4 or NaCl , as precipitating agents only. At every step of the various manoeuvres, the temperature, degree of acidity, time of exposure to this or that solvent, were found to be of significance. Two dangers had to be avoided, both of which are connected

TABLE I
Unitary product assayed against the standard powder

	BLOOD PRESSURE $\times$ STANDARD POWDER	OXYTOCIC ACTION $\times$ STANDARD POWDER	MELANOPHORE ACTIVITY $\times$ STANDARD POWDER
Product from experiment I.....	40-50	40-50	
Product from experiment II.....	45	50	
Product from experiment III.....		50	
Product from experiment IV.....	50	45-50	50
Product from experiment V.....	55-60	75	50

with acidity. Too long an exposure to an acid medium at a certain pH (which was not accurately determined at the time) caused a partial hydrolysis of the unitary substance so that the final product did not contain the active principles in the same ratio in which they exist in the standard powder, that is to say, a differentiation had taken place. Exposure to too low an acidity, as when a solution of a highly active unitary product was evaporated in an alcoholic solution in an air current at room temperature at a pH that was only slightly on the acid side of neutrality, resulted in the complete, or almost complete, loss of both pressor and oxytocic activity.

Avoiding the dangers referred to, I was able, with the assistance of Doctor Rouiller, to carry the purification of the ammonium sulphate product from $5 \times$ standard powder to 40 and $60 \times$ standard

powder, in five different trials in the year 1929. Table 1 gives the results of the trials. As a rule we started out with only relatively small quantities of the crude product, say, 5 to 6 grams, in order not to be delayed too long at any stage of the process. These earlier methods of purification have the disadvantage of giving only a low yield (10 per cent $\pm$) of the final amorphous unitary product with a value of 50 to 60 $\times$ the *standard powder*. To give an example; 5 grams of the crude ammonium sulphate powder yielded only 0.0464 of an end product evaluated at 50 $\times$ *standard powder*, for three activities, oxytocic, pressor and melanophore. In other experiments the final yield was somewhat better so that it is safe to state that a 10 per cent yield of highly active unitary substance can be obtained by the methods referred to. The missing active product is retained by several protein precipitates. These methods are now being replaced by others which will, it is hoped, lead to a higher yield of a still more powerful unitary product.

Similar tables could be given of numerous assays illustrative of the gradual rise in activity as purification of the crude product proceeded, as, for example, at stages when both pressor and oxytocic activities assayed at 20, 25, or 30, with the variations permissible in biological tests. A similar table could be constructed illustrative of the fact that certain chemical processes, more drastic in character, cause a marked differentiation of the activities of the final product, such that its unitary character is lost. In at least two instances the assay values of the final product for pressor and oxytocic activities lay so far apart that the divergence could not be laid to errors in the methods of assay. Thus, in one of these, the oxytocic activity was estimated at 100, while the pressor activity varied from 30 to 45 times that of the standard powder; in a second instance, the oxytocic activity was evaluated as varying from 120 to 140, while the pressor activity was lower than 50 and was finally estimated to be "about 25."

In this connection I may say that my experience leads me to believe that whenever the assay value for the pressor and oxytocic activities of a given precipitate are in close agreement, the precipitate will be found to exhibit all of the other physiological activities of the standard powder in the same ratio. It looks very much as

though the weakest link in the unitary molecule is that by which the oxytocic principle is joined to its associates.

In respect to the above assays, it may be stated that both cats and dogs were used in the blood pressure tests. Two of the products, IV and V, were tested on cats by three observers, Dr. K. K. Chen, who made use of the spinal cat, and Messrs. George E. Farrar, Jr., and S. J. Weinberg, whose studies were made with cats that had been anesthetized with phanodorn. The melanophore tests were carried out by Professor E. M. K. Geiling, who made use of frogs that had been bleached by exposure to sunlight, as also of the so-called silvery tadpoles, whose pituitary glands had been removed. The oxytocic tests were made by Dr. C. A. Rouiller.

WHAT IMPURITIES STILL REMAIN IN THE UNITARY PRODUCT WHOSE
ACTIVITIES ARE GIVEN IN TABLE 1?

The chief impurities, that is to say, the main physiologically inert constituents that contaminate the product, are proteins of albumose-like character. These, as I estimate, make up 50 per cent, at least, of the weight of the product. This conclusion was arrived at incidentally in the course of many experiments the purpose of which was not primarily the quantitative determination of inert proteins but rather the elaboration of profitable methods for their removal without alteration of the associated unitary principle. The methods employed in dealing with small quantities, as, say 60 mgm. more or less, of a unitary product with a titre of 40 to 60 times that of the standard powder, may not improperly be called micro-methods. Various inorganic precipitating agents as sodium chloride, sodium dihydrogen phosphate, silver acetate, and mercuric chloride, and numerous organic compounds were utilized in one way or another in these methods. Precipitates and also filtrates had always to be assayed in order to keep track of the unitary substance in its wanderings and to detect the first signs of its hydrolysis. It was often noted that the employment of one procedure or another yielded precipitates of protein character that had an assay value of only 10 to 20 per cent of that shown by the unitary product with which the experiments

were started. The rest of the active material was traceable to other precipitates and filtrates which very often had also retained more or less inert material of peptide nature.

The methods referred to have been given in barest outline, and, while they have not set me very far forward in the way of the further purification of the unitary principle, they have nevertheless served the purpose of showing that, taken at its best, it is still contaminated by inactive proteins to the extent of at least 50 per cent. The accuracy of this estimate will be checked later by comparison with the results obtained by methods of analysis that are destructive of the activities of the unitary principle but not of the chemical integrity of proteins.

A CRYSTALLINE COMPOUND PRESENT IN THE UNITARY COMPOUND
AND IN PITRESSIN

Aside from inert proteins and possibly a little ash, the unitary product appears to contain at least one other foreign substance in appreciable amount. This is a beautifully crystalline organic compound containing nitrogen and occurring in colourless, glittering prisms, with a melting point of $179^{\circ} \pm$. Dr. J. B. Koepfli has found that it is best recrystallized by dissolving it in the least quantity of absolute methyl alcohol, adding three volumes of acetone, and allowing the solution to stand in a closed flask. The base is soluble in water, in ethyl alcohol, in acids, in solutions of Na_2CO_3 and NaHCO_3 and does not evolve ammonia even on exposure of several days to ten per cent sodium hydroxide; it does not give the biuret, ninhydrin, Pauly, or Millon reactions. It is devoid of pressor, oxytocic, melanophore or antidiuretic activity. I have known of the presence of this base in my final products for more than two years and would here report that it is also present in appreciable amount in pitressin solutions, from which it is easily separated in crystalline form. The compound will be more fully described at a later date when its physiological properties shall have been more fully studied and when it shall have been determined whether it is a new constituent of the pituitary gland or only one of the previously isolated constituents of the animal organism.

SOME OTHER IDEAS IN RESPECT TO THE UNITARY SUBSTANCE VERSUS
THE PRESENT CONCEPTION THAT THE POSTERIOR PITUITARY
CONTAINS THREE OR MORE SEPARATE HORMONES

I

I would here point out that, although the unitary substance described above is still contaminated to the extent of 50 per cent with an inactive protein, not to speak of the very much smaller amounts of the crystalline base just described, there is no reason to believe that, given enough material, it will be impossible to remove these impurities so that a final unitary product will be obtained which comes close to being a chemical individual. My associate, Dr. Joseph B. Koepfli, and I are now engaged in this problem of the further purification of the crude unitary substance as obtained by the new copper method. Recently we have worked out a second step which involves the use of ethanolamine picrate as a precipitant. This agent was added to an alcoholic solution of the unitary principle and the picrates thrown out by it were decomposed in alcohol by addition of an alcoholic solution of free ethanolamines.³ This second step removes inactive crystalloids but does not greatly raise the activity of the product. A third step involves the use of mercuric chloride in an alcoholic medium for the partial removal of inactive proteins and, by this method, which calls for great attention to acidity, temperature, et cetera, in order to prevent inactivation or differentiation, we were able to carry the value of the unitary substance from 8 or 9 to from 30 to 35 times that of the standard powder and with a much better final yield than was obtained by fractional precipitations with various organic precipitants as described in the earlier sections of this paper. It is our intention to carry on the purification of the unitary substance along these lines.

³ I am under obligation to Dr. Ralph B. Trusler, Industrial Fellow at the Mellon Institute, for kindly supplying me with some of his "Triethanolamine," a mixture containing approximately 75 to 80 per cent of triethanolamine, 20 to 25 per cent of diethanolamine, and 0.5 per cent of monethanolamine.

II

The unitary substance, contaminated as it still is to the extent of 50 per cent or more by inert proteins, is readily decomposed into several active components. A small quantity of the unitary preparation V, whose assay value for pressor, oxytocic and melanophore activities is given in table 1, was dissolved in a few cubic centimeters of 0.25 per cent acetic acid and refluxed in a small apparatus for 1½ hours at boiling temperature. The solution was then transferred to a small, shallow bowl and evaporated to dryness in an air current. The residue contained some radiating fern-shaped crystals but was otherwise of amorphous character. It was exhausted with minimal quantities of water. About one quarter, more or less, of the residue remained undissolved as a gum-like mass which gives an intense biuret reaction and retains but little physiological activity. The few cubic centimeters of aqueous extract were precipitated cold and as rapidly as possible with a saturated solution of NaH_2PO_4 . The precipitate thus obtained was exhausted with 90 per cent phenol and the phenolic extract precipitated cold in the usual manner with acetone. When the resulting white powder was assayed against the standard powder it was found that a differentiation of activities had occurred. The values for vasopressor, oxytocic and melanophore activities were found to be respectively 75, 12 and 5. The melanophore activity was not determined on numerous frogs as it was apparently much lower than the oxytocic activity. Injection of 0.5 cc. of a 1:2000 dilution into the lymph sac of a bleached frog of average size caused no decided darkening of the skin in the course of an hour. Injection of one-twentieth cubic centimeter of the stock solution, containing 0.1 mgm. of the acetone precipitate per cubic centimeter, however, caused intense darkening of the bleached frog. The melanophore activity of the precipitate was, therefore, relatively small as compared with its vasopressor activity and could not have been more than $5 \times \text{standard powder}$. Expressed in terms of a ratio the relative activities of the acetone powder obtained after boiling the unitary substance with an acid are as 100:16:7, the first number representing the vasopressor, the

second the oxytocic and the third the melanophore activity of the powder.

Now product V had also been subjected in the final stage of its preparation to a single precipitation with NaH_2PO_4 , as had other specimens of the unitary substance, and this without differentiation of its physiological activities. It will be noted that preparation V, as shown in the above table, had an assay value of 55 to 60 for vasopressor, of 75 for oxytocic, and of 50 for melanophore activity. These values, taking into consideration the instability of our unitary substance, as also the experimental errors inherent in our biological assay methods, approach much more nearly to equality than is the case after the unitary substance has been hydrolysed.

Preparation IV, with a vasopressor value of 50, an oxytocic value of 45 to 50, and a melanophore value of 50, which values it will be seen stand in the relation to each other of 100:100:100, was also subjected to boiling with 0.25 per cent acetic acid for one hour and twenty-five minutes, but the aqueous extract of the dry residue after boiling was precipitated with saturated NaCl and the filtrate saturated with finely powdered NaH_2PO_4 . The NaH_2PO_4 precipitate thus obtained received a somewhat different treatment from that given to the analogous precipitate of product V, with the result that the final acetone product showed a higher protein content and a relatively lower content of active principles. Nevertheless, the differentiation in activity was fully as great in this case as in the experiment with preparation V. The vasopressor activity was assayed at 35, the uterine activity at 5, while the melanophore activity was not determined.

The results of the above experiments, it would appear to the writer, can only be explained on the supposition that when the unitary product is boiled with an acid of appropriate strength, it is split up into three or more physiologically active components. The methods utilized above are of value only in establishing this point and have now been discarded in part. NaH_2PO_4 can be used only in a limited way because of its high acidity, not to mention other disadvantages. I have come to the conclusion that this reagent will not precipitate any of the physiologically active

cleavage products of the unitary substance in the entire absence of proteins; in the presence of proteins, however, it always precipitates vasopressin to a much greater extent than the other active split products.

III

At this point it may be in place to say a word in relation to the antidiuretic action of the unitary substance, described above. I assume that this action is a property of this unitary substance just as it was shown definitely in 1923 to have been a property of two of the unitary products, described by myself and my co-workers, that had not been partially hydrolysed by repeated treatment with tartaric acid. This was proved in two hospitals⁴ by the efficacy of the products in reducing the output of urine in human diabetes insipidus.

The mouse-method for determining the antidiuretic value of a pituitary preparation which has been recently worked out by Professor O. S. Gibbs in this laboratory places at our disposal a means of deciding between two possible alternatives regarding this important action: (1) Is this action a property solely of the intact unitary substance? (2) Or is it a property that remains quite unaltered after hydrolysing, i.e., boiling the unitary substance for an hour or more with 0.25 per cent acetic acid? In the event that experiment should prove that only alternative (2) is tenable we should then have four known physiologically active split products of our unitary hormone, namely, (1) a vasopressor, (2) an oxytocic, (3) a melanophore, and (4) an antidiuretic substance. This question of a fourth active cleavage product will presently be put to the test of experiment in this laboratory.

IV

I can best summarize my present views on the question of post pituitary active principles by expressing them in the form of an equation. Let U represent the unitary molecule composed of three or four active principles united to each other in some form of union which yields as readily to hydrolysis as does the "peptide linkage" of numerous biochemical compounds.

⁴ Abel and Geiling: Jour. Pharmacol. and Exper. Therap., 1923, xxii, 317.

Let V = the pressor cleavage product, O = the oxytocic cleavage product, M = the melanophore cleavage product, Ad = the antidiuretic cleavage product, with the reservation made above, then

$$U + H' = V + O + M + Ad?$$

On this view we should be dealing with a unitary product which may provisionally be compared to a peptide though we can not be certain that it will give the biuret reaction when it shall have been isolated as a chemical individual, nor that the four (?) active cleavage products postulated above constitute the entire molecule.

OPINIONS IN RESPECT TO THE PROBABLE PHYSIOLOGICAL ACTIVITY
OF U—THE UNITARY PRODUCT—AND ITS CLEAVAGE PRODUCTS,
EXPRESSED IN TERMS OF THE STANDARD POWDER

I. The unitary product

It has been shown that the specimens of U described in this paper have an activity which, at its best, now varies from 50 to 60 times that of the international standard for V, O, and M (the three activities for which they are assayed) and that these activities are retained by separate cleavage products after U has been subjected to hydrolysis. Whether Ad, the antidiuretic property of U, can also be retained as a property of a fourth cleavage product remains to be demonstrated.

It was also shown that U, as above described, is still contaminated, as nearly as can be determined at the moment, with inactive proteins to the extent of 50 per cent. Let us assume that this 50 per cent of protein and also the crystalline contaminant referred to above are removable without alteration of the relative assay values of the vasopressor, oxytocic, melanophore and antidiuretic activities of U, an assumption that I have every reason to believe is well founded. In this case U would show an activity equal to $100 \times \text{standard powder}$ for each of its activities. After hydrolysis, however, each of its three known cleavage products, in case their respective molecular weights do not differ greatly

from each other, would have a value of 300 times the standard powder. In case of the existence of a fourth active cleavage product—an antidiuretic substance—this value would be proportionately enhanced and might reach the value of 400 times the standard powder for each of the assumed four cleavage products. The views outlined above receive strong support from the following considerations.

Seven years ago, as was stated at the beginning of this paper, there appeared from this laboratory a paper which showed that my pituitary tartrate of that time had an oxytocic action 1250 times stronger than that of the acid phosphate of histamine. Translating this value in terms of the international standard powder, which was not yet in use at that time, we find that the pituitary tartrate in question had an oxytocic value equal to 166 times that of the standard powder. In passing it may be remarked that the oxytocin (pitocin) of Kamm and his associates is stated to be "more than 150 times as potent as the international standard powdered pituitary." Certainly, then, this earlier product of this laboratory was fully as potent, if not more so, than the preparation called oxytocin. It was not claimed that my highly active tartrate was a pure substance. The work of the past two years has convinced me that it must still have contained an unknown amount of inactive proteins as also more or less of the crystalline base of unknown character referred to above. It also carried pressor and melanophore activities, though, as may now be admitted, not in proportion to its oxytocic activity. It is probably fair to assume that the inert protein plus the unknown base plus the fractions of pressor and melanophore cleavage products present in that highly active tartrate amounted to about 50 per cent of its weight. In that case a perfectly pure oxytocic cleavage product of U would have a potency of at least $166 \times 2 = 332 \times$ the standard powder, a value which is approximately of the same order of magnitude ($300-400 \times$ standard powder) as that which is provisionally attributed to each of the other cleavage products of U.

II. Pitocin

These calculations find support from a preliminary analysis of the oxytocin (pitocin) solutions of Kamm and his associates. It is admitted that this product contains only small amounts of the other active cleavage products of U, or rather perhaps a small amount of unhydrolysed and still intact U. It still, however, contains a very considerable amount of inert albumose-like substances which have not been quantitatively determined. The inert residue found when 210 cc. of pitocin were evaporated to dryness without injury was much less than that present in an equal volume of pitressin solution. It is of interest to know that the water-soluble part of the residue throws down only a negligible amount of oxytocin on saturation or near saturation with NaH_2PO_4 . Pitressin always falls out in greater proportion in presence of protein than does pitocin when subjected to this precipitating agent as was stated above.

The pitocin at my disposal was stated to have been "adjusted to contain in each cubic centimeter 10 international units" (1 international unit is equivalent to 0.5 mg. of the standard powder) and "less than $\frac{1}{2}$ unit of pressor activity." The dry residue of 5 cc. of the product weighed 1.4 mgm. on the micro-balance. This amount of residue, by definition, would have an oxytocic value equivalent to that of 25 mgm. of the standard powder. In other words the pitocin residue is, weight for weight, only 18 times more powerful than the standard and contains, according to my estimates, only about 6 and certainly less than 10 per cent oxytocin regarded as a chemical individual. It is safe to predict that when this principle shall have been isolated as a pure product from the above mixture it will fall in line with the above estimates in respect to its assay value which should be from 300 to 400 times that of the standard powder.

III. Pitressin

Secondly, my estimates receive strong support from the results of some preliminary analyses of the vasopressin (pitressin) solutions of Kamm and his associates which were made in the autumn

of 1928. Considerable quantities of this material which were stated "to contain less than one unit of oxytocic activity in each cubic centimeter" and which had been "adjusted to contain 20 pressor units in each cubic centimeter, one pressor unit being equal to 0.5 mgm. of standard powder," were purchased in Baltimore at that time. It will be recalled that vasopressin was believed to be "the substantially pure pressor principle (β -hypophamine) 80 times as potent as the international standard powdered pituitary," and that it was thought to be "responsible also for the diuretic-antidiuretic action of pituitary extracts."

The product was analyzed with the purpose of obtaining an approximate idea of the amount of inert material present in it. I regret to say that my records of the amount of dry residue present in each cubic centimeter of the commercial solution employed by me in 1928 have been lost and that I cannot here give its assay value per milligram of dry matter. I have, however, again determined the dry residue of a commercial pitressin solution which was purchased a few months ago. On concentrating 5 cc. in a tightly closed, dustless hood to about one-quarter cubic centimeter at room temperature and then drying *in vacuo* over CaCl_2 , and weighing on a micro-balance, it was found that the residue weighed 3.16 mgm. Anhydrous ether removed from it only 0.03 per cent of what appeared to be lipoidal matter, part of which was no doubt derived from the ether itself.

Inasmuch as the 5 cc. of pitressin had been adjusted to contain 20 pressor units in each cubic centimeter and since one pressor unit has a blood-pressure raising power equivalent to that exhibited by 0.5 mgm. of the international standard powder, it follows that the 5 cc. contained 100 pressor units equivalent to 50 mgm. of the standard powder in pressor value, and, since no physiologically active volatile agent was present, it again follows that the 3.16 mgm. of dry residue contained 100 pressor units and 1 mgm. 32 pressor units. Expressed in terms of equivalent weights of standard powder, as is the custom in this laboratory, rather than in terms of an arbitrary unit such as the "pressor unit" of Kamm, the dry residue of pitressin has a pressor value which is only 16 times greater than that of the standard powder. It may

not be out of place here to call attention to the fact that my still impure unitary product has a pressor activity which, at its best, is more than 300 per cent greater than that of commercial pitressin while still retaining its full equivalent of oxytocic activity.

Two hundred cubic centimeters of pitressin and more, the total acidity of which had been determined previously, were almost neutralized in the cold with $N/10$ NaOH, 50 mgm. of ammonium acetate were added, and with this as buffer material, the solution was evaporated in very large shallow petri dishes or watch glasses in a strong current of air. Preliminary experiments had shown that this manoeuvre could be carried out without injury to the vasopressor principle. When the residue was dry or very nearly so, it was taken up by repeated extraction with very small amounts of water and filtered. One is surprised to see how relatively large an amount of a denatured protein or proteins remains undissolved though considerable amounts of protein are also taken up by the water. This protein residue retains very little pressor activity.

To the aqueous extract finely powdered NaH_2PO_4 was added in the cold until no further increase of a stringy albumose-like precipitate was obtainable. The inactive proteins present in this precipitate, as also every trace of vasopressin, can be extracted from it with 90 per cent phenol. From the phenolic extract they are recovered as dry products by precipitation in the cold with organic solvents such as dry ether or acetone. The physiologically active dry powder gives the biuret reaction with great intensity, and while it has a very considerable pressor action it must nevertheless be assumed to contain a large amount of inert proteins in view of the well known power of saturated solutions of NaH_2PO_4 to precipitate substances of that character, as is well illustrated in the behavior of my unitary products toward this reagent. The further treatment of the products here described—a treatment involving what I may call micro-methods of precipitation, filtration and extraction—need not be given here. It may be remarked, however, that in the further course of purification of this and other lots of vasopressin, by means of these methods, still more inert protein than that first deposited could be removed.

The beautifully crystalline base already mentioned as contaminating my own unitary product was also found to be present in weighable amounts in the pitressin solutions and to constitute an appreciable fraction of the total dry residue.

In accordance with my experience with my own products I do not hesitate to say that the NaH_2PO_4 precipitate described above did not consist solely, or even mainly, of active constituents, but was composed to a large extent of inactive proteoses or peptides, as was the case with the analogous NaH_2PO_4 precipitate of my best unitary products, the pressor value of which varies from 50 to $60 \times$ the standard powder. We have then to subtract from vasopressin several inactive residues, all of which give the biuret reaction and which, as far as can be judged by their relative mass and without having been weighed, make up a very considerable proportion, surely not less than 50 per cent of the dry weight of the pitressin solutions. We have also to bear in mind, as will be more fully considered later, that pitressin solutions exhibit several distinct activities, each of which is no doubt confinable to a separate chemical entity by hydrolysis.

By the use of various solvents and precipitants as acetic acid, 90 per cent phenol, phenyl acetate, acetone, alcohol and ether, a little of a much more highly active vasopressin than has hitherto been described was obtained. This product was not tested for melanophore and antidiuretic activities as no effort was made to keep all of the activities of pitressin together, nor was I particularly concerned at the time with the problem of obtaining the vasopressin as a chemical individual. The small quantities of material at my disposal at the time would have militated against the successful outcome of the effort. Nevertheless, it is of interest to note that one of two still impure vasopressin preparations when injected intravenously in the minute dose of 0.0001 mgm. into two cats, anesthetized with phanodorn, and weighing respectively 2.30 and 2.35 kgm., raised the arterial pressure of each cat to the extent of 30 mm. of mercury. The second preparation caused an elevation of the arterial pressure of 26 mm. on being injected in a dose of 0.0001 mgm. thirty minutes after the injection of the first preparation. This considerable rise of pressure was in every

particular similar to that induced by a good pituitary preparation. Unfortunately, in the course of the four hours and more during which the experiments were carried on, only an approximate standardization could be achieved as we had not early enough injected comparable pressor doses of the standard powder. However, it was conservatively estimated that the two vasopressor products injected in doses of 0.0001 mgm. had an assay value of 240 and 210 respectively. As opposed to this high value, which is certainly far below the real value, it may be recalled that Kamm and his collaborators state that their "substantially pure pressor principle is 80 times as potent as the standard pituitary powder." I repeat that this active product was not a pure chemical individual.

The pitressin of Kamm and his coworkers possesses three physiological activities, two of which, at least, cannot, according to my own studies, belong to the vasopressor cleavage product itself. I have shown above that, after hydrolysis of the unitary principle, U, its pressor cleavage product can be obtained by a single precipitation and without further purification so that it is contaminated by only about 16 per cent of the oxytocic, and by much less of the melanophore cleavage product. Pitressin differs from my unitary product as regards its physiological activities (leaving out of consideration here the matter of inert by-products) in this respect: Pitressin is relatively free from oxytocic activity, but this activity is a function of my unitary product, where its value, relative to the other pituitary activities, is exactly equal to that of the standard powder. In a word, pitressin has vasopressor, antidiuretic and melanophore activities, which three activities stand in the relation to each other of approximately 1:1:1, as will be shown later, while my unitary substance carries four activities, vasopressor, antidiuretic, melanophore and oxytocic, whose relation to each other may be represented by the ratio 1:1:1:1. Three of these activities then are common to both pitressin and the unitary substance.

Here I may interrupt my argument to state that in my opinion the reason that Kamm and his coworkers have always found a certain per cent of oxytocin in their vasopressin and, contrariwise,

a certain though smaller proportion of vasopressin in their oxytocin is best explained on the theory of an incomplete hydrolysis of the original unitary substance by their treatment with acetic acid and ether, so that a small amount of unaltered unitary substance remains undecomposed. In support of this conjecture I would call attention to the fact that pitocin solutions exhibit a melanophore activity which is often very marked when small or medium sized frogs are injected with 0.5 cc. of a 1:50 dilution. This amount represents no inconsiderable fraction of the melanophore principle present in 0.5 cc. of a 1:4000 or 1:5000 dilution of pitressin, at which dilutions pitressin is effective in expanding melanophores, but hardly more so than a 1:50 dilution of pitocin. No study of the antidiuretic activity of pitocin has yet been made with sufficiently large doses to exclude its presence.

The Antidiuretic Action of Pitressin

As we have seen, Kamm and his associates claim that the pressor principle is responsible for the antidiuretic action of pituitary extracts, and their words can only be interpreted as meaning that both the pressor and antidiuretic activities are properties of a single chemical entity. In accordance with this view physicians have been advised "that pitressin is indicated especially in diabetes insipidus." That pitressin solutions have an antidiuretic action cannot be questioned, I believe, in view of the corroborative results recently obtained in this laboratory by Professor O. S. Gibbs (4) in tests that were made by his new mouse method. He makes the following statements in respect to his findings:

I have roughly tested the activity of oxytocin, and of vasopressin kindly supplied me by Dr. Kamm. As will be seen, oxytocin contains little if any antidiuretic activity while the vasopressin is active. A sufficient amount of data is not available to determine accurately whether the antidiuretic property is strictly in relation to the pressor property; so far no striking distinction has appeared in the few tests I have conducted.

It is admitted then that pitressin solutions carry antidiuretic activity in practically the same proportion as vasopressor activity, as was first shown by Bugbee (3) in 1928.

The belief of Kamm and his associates that both antidiuretic and vasopressor activities are properties of one and the same preformed hormone finds however no support in the studies of Bijlsma, Burn and Gaddum (5) who have "shown that the antidiuretic effect is not due either to the pressor or the oxytocic principle," and who suggest that the antidiuretic effect is due to a third principle. From my point of view this third principle would be merely a third cleavage product of the unitary principle and not a preformed constituent of the pituitary gland. I hope in due season to put this theory to a practical test by hydrolysing the unitary principle and searching for a separate antidiuretic cleavage product.

The Melanophore Activity of Pitressin

I have already given it as my opinion that this activity can be confined by hydrolysis of U to a separate cleavage product. Rowe (6) has given an account of the contradictory opinions of previous investigators as to whether the melanophore activity is a property of the oxytocic or of the vasopressor "principle." The work of this author seems to me to prove, and our studies in this laboratory corroborate his conclusions, that oxytocin (pitocin) solutions do not stimulate frog melanophores except when injected in dilutions of 1:50. Vasopressin (pitressin) solutions, however, stimulate frog melanophores very decidedly in dilutions of 1:4000 and on this point, also, I am in entire agreement with Rowe (6) as also with his suggestion "that the melanophore constituent may be a separate principle," with the reservation, however, that it is to be designated a *cleavage product* of the unitary principle and *not a preformed principle*.

Rowe (6) finds that the melanophore stimulating action of vasopressin solutions "is apparently to an appreciable degree less than that of a pituitary extract of the same potency," and states further, "that the difference in favor of the pituitary extract seems to be about 20 to 25 per cent." His discussion in respect to the difficulties inherent in the methods of testing melanophore activity, from the point of view of quantitative measurements, is eminently fair and unprejudiced. I cannot help regretting how-

ever that he did not also make use of hypophysectomized tadpoles in addition to bleached frogs and thus have two standards for comparison. In view of the very high melanophore activity of vasopressin solutions I am not yet prepared to accept his conclusion that such solutions are actually less powerful to the extent of 20 or 25 per cent than pituitary extracts of equal pressor activity. The results obtained with bleached frogs should be checked against those obtained with "silvery tadpoles" before we can be quite sure that the assumed divergence in melanophore and vasopressor potency of pitressin solutions is not due to the quantitative inadequacy of our present methods for testing these activities.

It has been shown that vasopressin (pitressin) solutions are not solutions of a "substantially pure pressor principle" as Kamm and his associates (3) believed them to be. On the contrary such solutions represent only a more or less refined pituitary extract from which the oxytocic principle has been largely or almost entirely removed as the case may be. In view of the preliminary studies outlined above I infer that the oxytocin has been removed by the hydrolytic action of the acetic acid employed by Kamm. What is left in the pitressin solutions still contains the antidiuretic and melanophore activities in addition to the vasopressor activity as has already been pointed out. These three activities of pitressin stand in a relation to each other that certainly varies little from the ratio 1:1:1. That these three physiological activities, two of which can be confined to separate chemical entities after hydrolysis (and the third of which is likely also to be a function of a third cleavage product), should all be found in this singular ratio in the same ether precipitate, if they originally existed as three separate principles, is rather surprising.

My work of the past two years convinces me that a complete or even partial differentiation of separate principles can only be effected by organic solvents when the unitary principle has first undergone a more or less complete hydrolysis. I suspect therefore that pitressin solutions do not contain two or three separate, preformed principles, but that their physiologically active constituent is in reality a larger molecule with multiple activities and represents what is left of the unitary principle, U, as the result of a partial hydrolysis leading to the removal of the most easily

separable cleavage product—oxytocin. From this point of view pitressin differs from my unitary principle in that it is the carrier of three activities, vasopressor, antidiuretic and melanophore, while the unitary principle carries four activities: vasopressor, antidiuretic, melanophore and oxytocic, standing in the relation to each other as 1:1:1:1. Inasmuch as the three activities of pitressin solutions stand in relation to each other in about the ratio of 1:1:1, it is assumed that these solutions originally contained an undecomposed molecular-complex which differs physiologically from my unitary principle only in having lost its attached oxytocin as the result of a partial hydrolysis. During sterilization of pitressin with application of heat (if this procedure was employed) the assumed unstable active molecule would naturally fall apart, more or less completely, with the appearance of three active fragments. This hypothesis, as to the unitary, or largely unitary, character of pitressin as originally precipitated by Kamm and his associates, can readily be put to the test of an experiment in which, however, all further hydrolysis must be carefully avoided.

However this may be, it remains a fact that the vasopressin solutions of Kamm and his associates contain a very high percentage of inert material, as was shown above, in addition to the carrier or carriers of its physiological activities. In consequence there can be little doubt that if a single, physiologically active, cleavage product, as say pure vasopressin, were to be isolated from this mixture, it would be found to have an activity of 300 to 400 $\times$ the standard powder, in agreement with the estimate that has already been made in preceding pages from other points of view.

The facts adduced in the preceding pages and the concepts that have been formulated relative to them may best be recapitulated in the form of equations whose symbols have already been defined. The on the right hand side of equations 1 and 2 is meant to designate the unitary character of the enclosed product.

$$(1) \text{ U, the unitary principle in a pure state } = \boxed{\text{V-O-M-Ad}}$$

$$(2) \text{ "Pitressin," as first precipitated, probably } = \boxed{\text{V-M-Ad}}$$

$$(3) \text{ U} + \text{H}' = \text{V} + \text{O} + \text{M} + \text{Ad}?$$

$$(4) \text{ "Pitressin"} + \text{H}' = \text{V} + \text{M} + \text{Ad}?$$

REMARKS IN RELATION TO THE "PITUITARY TARTRATE"
OF 1923

In the autumn of 1926 and the winter months of 1926-1927 Dr. M. L. Tainter very kindly repeated my earlier work of 1920-1923. It was found that the mercuric chloride method as applied to the fresh posterior pituitary was serviceable only when 100 grams of fresh material were worked up at a time and much less so with larger quantities. Tainter's work, which was not published, showed conclusively that while the active product obtained by this method was at first unitary in character, it later became differentiated when it was treated with strong acids. One of his products, a tannate containing much NaCl, was repeatedly exhausted with a saturated solution of tartaric acid in 93 per cent alcohol and precipitated with ether, the precipitate being again taken up in the tartaric acid solution and again precipitated with ether, the process being repeated five times. The final precipitate had now become so far differentiated as to be purely pressor in character. On the other hand it was found that the filtrates, on proper treatment, yielded an oxytocin that was devoid of pressor action. These preparations were, however, still contaminated by inert proteins and crystalloids. These unpublished findings of Dr. Tainter were obtained before the appearance of Kamm's paper in February, 1928, and they taught me to be more careful in future efforts to raise the unitary product to a higher level without differentiation of its activities.

It may be thought that Tainter's prolonged treatment of his tannate with tartaric acid, alcohol and ether, served only to facilitate the separation of two preëxistent chemical individuals from each other and that there is little justification for the belief that a unitary product was hydrolysed by that treatment. Against this view it may be urged that acids are not required for the separation of cleavage products once these have been liberated from the unitary product on boiling it with 0.25 per cent acetic acid. It is also hard to understand why a unitary product can be precipitated out of an alcoholic solution by means of a mixture of ethanolamines, if the various "active principles" exist as preformed individuals, especially in view of their ready solubility in

alcohol. Lastly, various acids, under proper safeguards, are repeatedly used in the preparation of my unitary product without differentiating it—a result that could not be attained, it appears to me, if the various activities of post-pituitary extracts are originally confined to several individuals which inevitably undergo a separation when they are subjected to the mere solvent action of acids and organic fluids.

References to the papers of those investigators who have criticized the work of myself and my associates that appeared in 1923 will be found in the paper of Kamm and his coworkers. The criticisms of our work there summarized are justified only in so far as they concern the final tartrate with an oxytocic titre of 1250 times that of the acid phosphate of histamine, or, expressed in terms of the standard powder, which was not then available, with an oxytocic value 166 times greater than that of the present standard powder. The criticisms do not hold for the tartrates of lower assay values which were still completely unitary in character. On page 311 of my paper with Geiling and Rouiller will be found the results of experiments, in which a tartrate with an oxytocic titre of 160 times that of histamine acid phosphate was matched against an aqueous extract of the posterior lobe, for oxytocic, pressor and diuretic values. I do not recall the concentration of the aqueous infundibular extract used in the experiments, if indeed it was thought worth while to determine it, but that there was a singular correspondence between three physiological activities of the tartrate and of corresponding volumes of the aqueous extract employed can not be doubted. These early results which were obtained with the tartrate referred to above are fully confirmed by the facts presented in this paper. I can only regret that these experiments of 1923 were not given in the form of a table with data as to the concentration of the aqueous infundibular extract employed in place of the present extract of the standard powder.

In conclusion I would observe that I have not hitherto taken into consideration either the hyperglycemic action or the lipid altering agency of post-pituitary extracts and the connection of these activities with the unitary principle and its cleavage products.

GENERAL SUMMARY AND CONCLUSIONS

1. I have endeavored to show in this paper that it is possible to prepare a unitary pituitary product in the form of a colorless dry powder readily soluble at room temperature in 0.25 per cent acetic acid which retains all of the known physiological activities of the international standard pituitary powder and in the same relative proportions in which they exist in the standard powder, but with such an increase in potency that the product is from 50 to 60 times as powerful as the standard for the three activities for which it has thus far been assayed.

2. The unitary product as described is still contaminated to the extent of about 50 per cent with inert albumose-like proteins and also contains a small amount of a beautifully crystalline physiologically inactive base which has not as yet been identified. This base is also present as a constituent of that mixture of inert and active ingredients known as vasopressin, or pitressin, which, while carrying less than 10 per cent of oxytocic activity, has a vasopressor activity that is about one third that of my admittedly still impure unitary product at its best.

3. The unitary product can be hydrolysed by boiling for an hour or more with a solution of an acid whose pH is equal to that of a 0.25 per cent acetic acid, with the result that several physiologically active but chemically different cleavage products can be roughly separated by a few simple operations.

4. Taking into consideration the amount of inert components still present in the unitary substance with a value of 50 to 60 times the standard powder for each of its activities, and the ease with which it can be converted by hydrolysis into a number of physiologically active and dissimilar cleavage products, it seemed worth while to venture a probable estimate of the ultimate or maximum assay value of the cleavage products considered as chemical individuals. The calculations made receive support from a critical analysis of vasopressin (pitressin) and oxytocin (pitocin). It is estimated, that, considered as pure cleavage products, the pressor principle, oxytocin, and the rest, will have an assay value of *300 to 400 times the standard powder*, on the assumption that these cleavage products have molecular

weights which do not differ too widely from each other. This estimate receives strong support also from an earlier paper from this laboratory in 1923 in which a pituitary tartrate was described which at the lowest assay was equal in oxytocic potency to 1250 times its weight of the acid phosphate of histamine, or, translated into modern terms, equal to *166 times its weight of the present standard powder*. The work of the past two years shows that my pituitary tartrate of 1923 could well have been contaminated by non-oxytocic, biuret-yielding proteins plus an unknown amount of non-oxytocic, physiologically active constituents to the extent of 50 per cent, more or less. On this assumption we may set down the probable assay value of pure oxytocin to be in the neighborhood of $166 \times 2 = 332 \times$ that of the standard powder.

5. Incidentally, a critical analysis based partly on preliminary studies of my own, and in part on the cited work of others, was made of the pitressin solutions of Kamm, Aldrich, Grote, Rowe and Bugbee from which the following conclusions were drawn: These solutions do not contain a single physiologically active principle, vasopressin, but carry three different activities in about equal proportion, namely, (1) vasopressor, (2) melanophore, (3) antidiuretic. Two at least of these activities, the vasopressor and the melanophore, can be confined to dissimilar split products by hydrolysis, and this may be found to be true also of the antidiuretic property. It is suggested that the three activities are confined, at the time when pitressin is precipitated by ether, to a single molecule representing the larger part of the unitary substance after a partial hydrolysis in which oxytocin alone was for the main part removed. It was also shown that pitressin is contaminated, to a very large extent, with albumose-like inactive proteins, and to a smaller extent with a crystalline base of at present unknown character. While the amount of these impurities was not quantitatively determined it is, nevertheless, estimated to constitute at least 50 per cent of the dry weight of pitressin solutions.

Summing up these points it may be said that, while Kamm and his associates are to be credited with having separated oxytocin as a cleavage product of the unitary principle, the conclusion

is nevertheless unavoidable that their pitressin, as at present constituted, is a heterogeneous mixture of inert and active products and therefore far removed from being a solution of "the substantially pure pressor principle" as they supposed it to be. It constitutes in reality only a more or less refined pituitary extract which has been deprived of its oxytocic activity while retaining the three other activities characteristic of such extracts together with a small amount of the undecomposed unitary substance.

Since pitressin contains so large an amount of inert material it becomes at once apparent that its several active constituents, once they have been isolated, will each approach more or less closely in assay value to the probable estimate given above, namely, 300 to 400 times that of the standard powder. I am fortified in this belief by the results of a preliminary analysis of the product, in the course of which I obtained a pressor principle which, while it was still far from being a chemical individual, nevertheless had a pressor value that was certainly not less than 240 times that of the standard powder. Kamm and his associates state that pitressin has a pressor value equal to 80 times that of the standard powder. I estimate that the pitressin solutions at my disposal in 1928 could not have contained even as much as 10 per cent of pure vasopressin.

The commercial oxytocin of Kamm and his associates has also been shown above to be far removed from being a chemical individual and is estimated to contain less than 10 per cent of the pure principle.

6. As time and material at my disposal permit, I hope to carry on my work on the further purification of the unitary product described in this paper. Very naturally, its physiologically active cleavage products will also be studied. I can see no reason for believing, given sufficient material, that the remaining 50 per cent of inert material can not be removed from it, without, at the same time, disturbing the relation of its several physiological activities to each other, that is to say, without injuring its unitary character. Preliminary trials indeed have recently yielded small quantities of the unitary substance that approximated to the value

of $100 \times$ the standard powder. If further experiments confirm the results of these preliminary trials, it may possibly turn out that the purified unitary substance with a value for all activities in the neighborhood of $100 \times$ the standard powder may be induced to fall out of solution as a crystalline chemical entity. It will probably be regarded as unlikely that this eventuality will ever become a reality, but this outcome is worth striving for in view of the importance of the problem.

In any event it must be apparent that with increasing purification of the unitary product, its physiologically active cleavage products, vasopressin, oxytocin and the others, must show a correspondingly higher assay value versus the standard powder. It will also be admitted that the chances of crystallizing these active cleavage products, which my studies lead me to believe are all of non-protein character, become increasingly favorable as the unitary principle approaches more closely to individuality.

My sincere thanks are due Professor E. M. K. Geiling, Drs. C. A. Rouiller, M. L. Tainter, K. K. Chen, O. Wintersteiner, J. B. Koepfli and M. Rosenfeld, the Messrs. Weinberg, Farrar and Merritt and Miss de Lawder, each of whom has given me valuable assistance at one time or another during the past two years, sometimes in the chemical operations and again in making hundreds of bioassays of precipitates and solutions. Grateful acknowledgment is also made to the officials of the Research Laboratory of Armour and Company of their generous donation of costly material and to the Carnegie Corporation of New York for a liberal grant, without which it would have been impossible to carry on the work outlined above or to continue that now in progress in this laboratory on other hormones.

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